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Gene Expression Patterns 4 (2004) 389–395

GEP

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The proliferation associated nuclear element (PANE1) is conserved between mammals and fish and preferentially expressed in activated lymphoid cells

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Received 15 August 2003; received in revised form 11 January 2004; accepted 12 January 2004

Available online 27 February 2004

Abstract

The proliferation associated nuclear element (PANE1) had been identified in a screen for genes activated in mouse mammary epithelium transformed by stabilized β-catenin. We have now cloned the human and zebrafish orthologs, analyzed their expression and expressed them ectopically in tissue culture cell lines. PANE1 consists of 180 amino acids and displays 38% conservation between man and zebrafish. Expression of the human *PANE1* gene was detected preferentially in immune cells including leukemias and lymphomas, tumor tissues and tumor derived cell lines. In B- and T-cells PANE1 RNA was only detected after the respective cell types were activated either in vivo or in vitro.

Keywords: Proliferation associated nuclear element; Zebrafish; β-Catenin; Leukemia; Lymphoma; PANE1

1. Background

The proliferation associated nuclear element (*PANE1*) gene was originally identified as a gene highly expressed in β-catenin transformed mouse mammary tissue (Renou et al., 2003). PANE1 consists of 180 amino acids and no known motifs have been identified. Studies with PANE1 expression vectors in tissue culture cells have revealed that PANE1 is preferentially present in the nucleus in non-confluent cells and is excluded from the nucleus during cell division or upon confluence (Renou et al., 2003).

PANE1 is abundant in mammary squamous metaplasias that were induced by experimentally stabilized β-catenin in secretory epithelial cells. However, it was not known whether PANE1 expression had been induced in the epithelial compartment or was the result of the immune response that accompanied the squamous transformation. It was hypothesized that the immune cells, if responsible for the expression of PANE1, might be predominantly of the activated type. The second possibility was that the *PANE1* gene was a direct downstream target for the stabilized β-catenin in the epithelial compartment.

Three experimental approaches were applied to gain additional information on PANE1. First, the human and zebrafish ortholgs were isolated. Second, the expression pattern of PANE1 was explored in various human tissues and tissue culture cells. Third, a direct comparison of the cellular behavior of human and zebrafish PANE1 was performed in tissue culture cells.

2. Results and discussion

2.1. Isolation of the proliferation associated nuclear element gene from man and fish

Mus musculus PANE1 cDNA and protein sequences were blasted against *Homo sapiens*, *Rattus norvegicus*, *Anopheles gambiae*, *Drosophila melanogaster*, *Fugu rubripes*, *Danio rerio*, *Caenorhabditis briggsae* and *Caenorhabditis elegans*

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doi:10.1016/j.modgep.2004.01.008

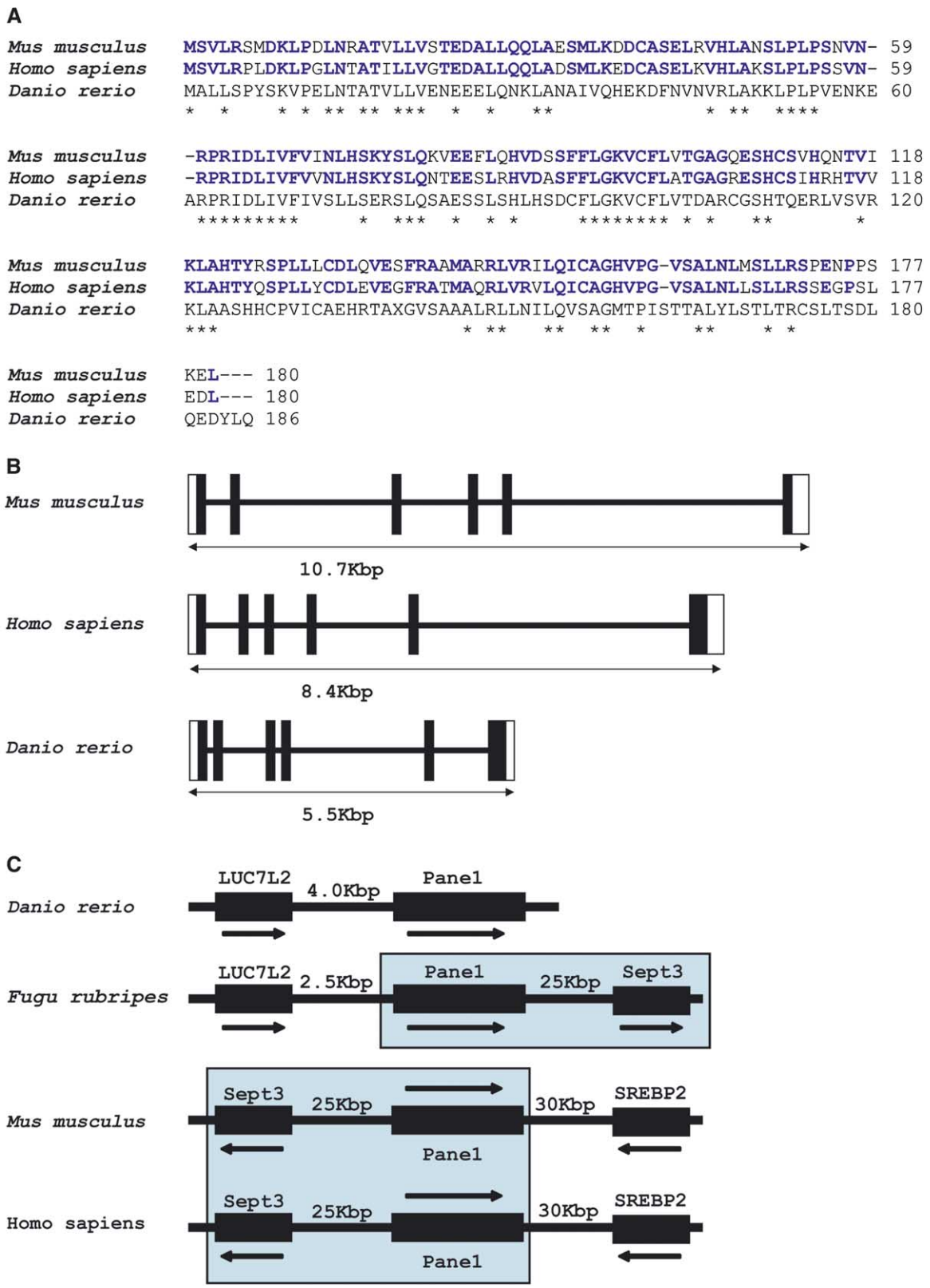


Fig. 1. Protein alignment and genomic structure of PANE1 from *Mus musculus*, *Homo sapiens* and *Danio rerio*. (A) While the protein sequences for the human and mouse PANE1 are highly conserved (blue) the zebrafish PANE1 retains 38 and 42% conservation of the amino acid sequence when compared to human and mouse sequences, respectively. (B) PANE1 genomic loci from mouse, human and zebrafish. The genomic organization has been conserved in three species. The initiation and termination codons are located within the first and last exon, respectively. (C) Representative neighboring genes that flank PANE1 in zebrafish, fugu, mouse and human genomes. This illustration is not to scale, but it demonstrates the orientation of several known genes that represent

genomic and translated protein databases and against all of the cDNA repositories at NCBI. Positive results were only returned for vertebrate hosts (*H. sapiens*, *R. norvegicus*, *F. rubripes* and *D. rerio*). While full-length clones were identified for *H. sapiens* and *R. norvegicus*, *F. rubripes* and *D. rerio* blasts returned only small portions of predicted translated protein sequences. Putative PANE1 genomic data from *D. rerio* were used to identify in silico sequences with an open reading frame (ORF). Subsequently, the entire PANE1 ORF was amplified by RT-PCR from cDNA prepared using mature zebrafish. The human PANE1 cDNA was isolated as a full-length clone from a colon adenocarcinoma. The protein sequences for PANE1 from zebrafish (AY243536) and mouse (AY243537) were 42% conserved (Fig. 1A). Moreover, the PANE1 genomic structure with its six exons was conserved between mammals and fish (Fig. 1B). Although PANE1 is present in mammals and fish, no ortholgs were found in *A. gambiae*, *D. melanogaster*, *C. briggsae* and *C. elegans*. If these species contain PANE1 orthologs, they are not sufficiently conserved to be detected using current search algorithms.

The genomic loci for human, mouse, zebrafish and fugu PANE1 were analyzed (Fig. 1C). The mammalian loci were highly conserved and the *PANE1* gene was flanked by known genes encoding Septin 3 upstream, and the Sterol Regulatory Element Binding Protein 2 (SREBP2) downstream. The arrangement of genes in this locus differed in fugu. While the *PANE1* and *Sept3* genes in mouse and man were arranged in a head-to-head orientation, the two genes in fugu were arranged in a head-to-tail orientation. No sequences resembling the *SREBP2* gene were found for the fugu or zebrafish genome in the vicinity of the *PANE1* gene. Instead of the *SREBP* gene, both fugu and zebrafish loci contained upstream sequences identified as LUC7L2 (yeast LUC7 like 2). In zebrafish the genomic sequences around the *PANE1* were not extensive enough to generate a link to the *Sept3* gene.

2.2. Expression of the PANE1 gene

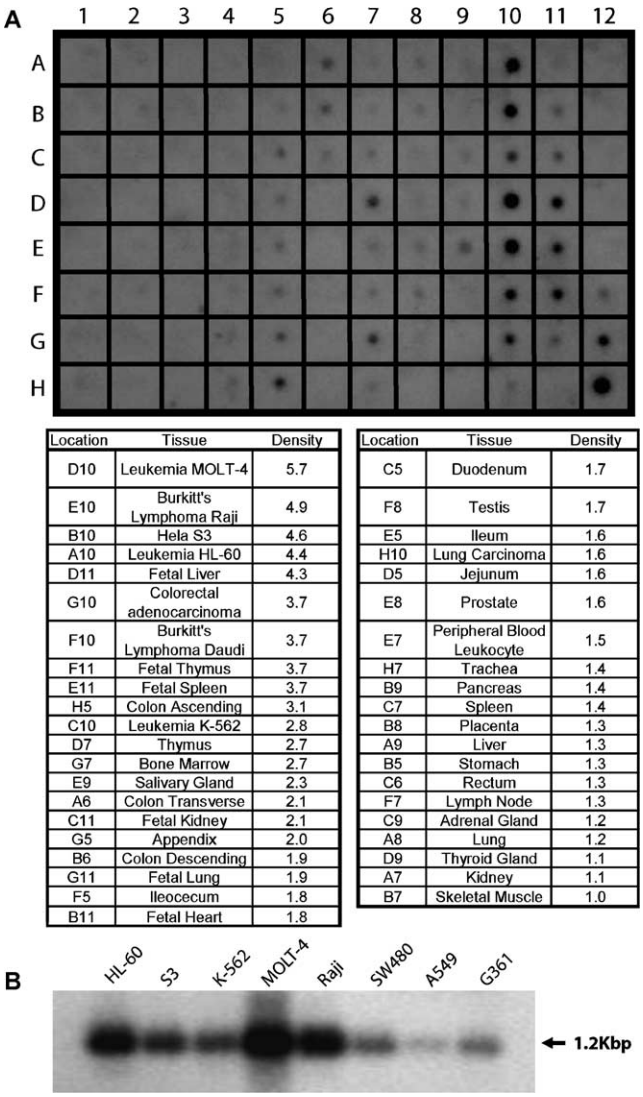
Expression of the *PANE1* gene was established in silico and through RNA analyses. The UNIGENE clusters Hs.208912, Mm.23596 and Dr.14405 corresponded to the *PANE1* gene, and an enrichment of PANE1 sequences was observed in several libraries. The highly enriched expression in tumor tissues and tumor cell lines (including lymphomas and leukemias) within the cDNA libraries initially prompted the effort to further explore this gene. Out of 145 unique clones from the human UNIGENE cluster, 116 (80%) were from a cancer related tissue or cell type (data not shown). In the Hs.208912 cluster several classes of tissues and cell types were identified. The first class encompassed cDNA representing whole lesions as often noted by the 'oma' suffix without the 'cell line' designation. The second class included specific cell lines representing transformed cell types. The third class covered normal tissues that express PANE1, and several of these tissues were also represented in the Mm.23596 and Dr.14405 clusters. UNIGENE cluster Hs.208912 demonstrated an enrichment of PANE1 sequences in tumors, leukemias and tumor cell lines from defined lineages. Moreover, high expression of the *PANE1* gene in *H. sapiens* has been identified in the lower gastrointestinal tract and several other tissues, including samples from fetal development.

A quantitative analysis was performed in order to eliminate the bias from the bioinformatic screen that is inherent due to the limits of EST sequences in the databases. A Multiple Tissue Expression Array (MTE) containing 75 human tissues and cell lines was probed with a human PANE1 cDNA (Fig. 2A, image). The relative spot densities demonstrated a tissue-preferential PANE1 expression pattern (Fig. 2A, table). Tissues with high expression included fetal liver, thymus and spleen as well as lymphoid cells and tumor cell lines including B- and T-cell leukemias. Little expression was observed in the majority of adult tissues. To further validate the specificity of PANE1 hybridization, a multiple tissue Northern blot (MTN) was probed (Fig. 2B). A single RNA transcript with the predicted size of 1.2 kb was identified in several cancer cell lines and the expression pattern mirrored that seen in the MTE.

2.3. PANE1 is expressed in activated B- and T-cells

Both the in silico and RNA analyses demonstrated that the *PANE1* gene was preferentially expressed in lymphoid cells and tumors, which in turn suggests a role in the immune response. To further explore this, PANE1 expression was analyzed in primary B- and T-cells before and after their activation (Fig. 3). While no PANE1 mRNA was detected in resting B- and T-cells, PANE1 transcripts were detected in T-cells that had been activated with PHA, EBV-immortalized B cells and primary B cells stimulated through CD40 ligation in the presence of interleukin-4 (Fig. 3A). This result demonstrated that B- and T-cell activation tightly regulates *PANE1* gene expression. To determine whether PANE1 transcripts are simply present in all tumor cell lines, a cDNA panel representing a variety of non-lymphoid tumor cell lines was analyzed (Fig. 3B). No expression was detected in any of the cell lines. Moreover,

the similarities and divergence within these loci. Gene sequences shown are *PANE1*, *Septin 3* (Sept3), *Sterol Regulatory Element Binding Protein 2* (SREBP2) and *LUC7L2* (yeast LUC7 like 2). Arrows indicate orientation of the respective genes, and the boxed regions indicate similar neighboring genes near the PANE1 loci. Note the difference in head-to-tail orientation of the *Sept3* gene when mammalian loci are compared to fugu. The current length of genomic sequence available for the zebrafish PANE1 locus was not sufficient to determine if Sept3 could be localized to this region.



Location	Tissue	Density
D10	Leukemia MOLT-4	5.7
E10	Burkitt's Lymphoma Raji	4.9
B10	Hela S3	4.6
A10	Leukemia HL-60	4.4
D11	Fetal Liver	4.3
G10	Colorectal adenocarcinoma	3.7
F10	Burkitt's Lymphoma Daudi	3.7
F11	Fetal Thymus	3.7
E11	Fetal Spleen	3.7
H5	Colon Ascending	3.1
C10	Leukemia K-562	2.8
D7	Thymus	2.7
G7	Bone Marrow	2.7
E9	Salivary Gland	2.3
A6	Colon Transverse	2.1
C11	Fetal Kidney	2.1
G5	Appendix	2.0
B6	Colon Descending	1.9
G11	Fetal Lung	1.9
F5	Ileocecum	1.8
B11	Fetal Heart	1.8

Location	Tissue	Density
C5	Duodenum	1.7
F8	Testis	1.7
E5	Ileum	1.6
H10	Lung Carcinoma	1.6
D5	Jejunum	1.6
E8	Prostate	1.6
E7	Peripheral Blood Leukocyte	1.5
H7	Trachea	1.4
B9	Pancreas	1.4
C7	Spleen	1.4
B8	Placenta	1.3
A9	Liver	1.3
B5	Stomach	1.3
C6	Rectum	1.3
F7	Lymph Node	1.3
C9	Adrenal Gland	1.2
A8	Lung	1.2
D9	Thyroid Gland	1.1
A7	Kidney	1.1
B7	Skeletal Muscle	1.0

Fig. 2. Multiple tissue expression array analysis of PANE1 transcript distribution. A multiple tissue array was used. (A) A radiolabeled cDNA corresponding to the human *PANE1* gene was hybridized to a polyA multiple tissue expression array (MTE) containing 75 human tissues. The fetal spleen, thymus and kidney are pooled samples from 16 to 32 weeks, the fetal lung is pooled from 20 to 30 weeks, the fetal heart is pooled from 21 to 37 weeks and the fetal liver was pooled from 22 to 40 weeks of age. The table below the array displays relative spot densities with the lowest signal above background normalized to 1.0 in descending order according to expression levels. The array location for each tissue in the expression profile also appears in the table to allow a quick visual verification of the spot densities. No signals above background were obtained for whole brain [A1], cerebral cortex [B1], frontal lobe [C1], parietal lobe [D1], occipital lobe [E1], temporal lobe [F1], paracentral gyrus of cerebral cortex [G1], pons [H1], left cerebellum [A2], right cerebellum [B2], corpus callosum [C2], amygdala [D2], caudate nucleus [E2], hippocampus [F2], medulla oblongata [G2], putamen [H2], substantia nigra [A3], nucleus accumbens [B3], thalamus [C3], pituitary gland [D3], spinal cord [E3], heart [A4], aorta [B4], left atrium [C4], right atrium [D4], left ventricle [E4], right ventricle [F4], interventricular septum [G4], apex of heart [H4], esophagus [A5], fetal brain [A11], bladder [C8], uterus [D8] and mammary gland [F9]. (B) Human tumor multiple tissue polyA Northern blot. The tumors represented on the Northern blot are the promyelocytic leukemia HL-60, Hela S3, Chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Raji type Burkitt's lymphoma, colorectal adenocarcinoma SW480, lung carcinoma A549 and the melanoma G361 cell lines listed in the order that they appear.

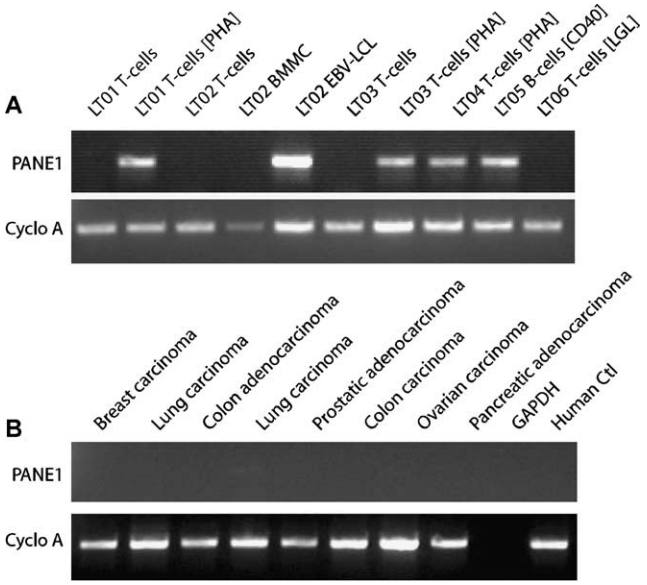


Fig. 3. RT-PCR analysis of PANE1 expression. (A) Primary lymphocyte cultures were monitored for PANE1 expression. LT01, LT03 and LT04 were the designations for resting primary T-cell samples and they were activated in vitro using phytoheamoglutinin (PHA) + IL-2. LT02 was the designation for a donor sample set that contained resting T-cells, BMMC, and EBV-LCL. Samples from donor LT05 were in vitro activated B cells. LT06 was a sample of T-cells from a large granular lymphocyte leukemia (LGL) patient. Only the cells that have been activated in vitro express a PANE1 transcript as demonstrated in the PANE1 panel after normalization with cyclophilin A as shown. (B) A multiple tumor cDNA panel was screened for PANE1 expression under the same conditions used for the B- and T-cells after normalization to cyclophilin A. The cDNA panel represents a poorly differentiated mammary carcinoma, poorly differentiated metastatic lung carcinoma, moderately differentiated adenocarcinoma from the colon, poorly differentiated lung carcinoma, grade IV adenocarcinoma, moderately differentiated colon adenocarcinoma, undifferentiated carcinoma isolated from a primary ovarian carcinoma and a poorly differentiated pancreatic adenocarcinoma listed as they appear. A significant level of expression was not detected a level comparable to activated B- and T-cells.

several breast cancer cell lines were negative for PANE1 (SK-BR-3, BT-474 and MD-MB-231 lines; data not shown).

2.4. Ectopic expression of zebrafish and human PANE1

To establish whether the unique sub-cellular distribution of mouse PANE1 was conserved in zebrafish and human, the respective cDNAs were cloned into expression vectors, along with an in-frame C-terminal myc tag. Transiently transfected HeLa cells were probed with anti-myc antibodies and subjected to immunofluorescent detection. Zebrafish and human PANE1 did behave in a manner consistent with the mouse protein as determined by the nuclear localization in non-confluent cultures (Fig. 4A,D) and an absence of expression within the nucleus upon confluence of the culture (Fig. 4B,E) or during cell division (Fig. 4C,F). Upon selection of PANE1 expressing cells to

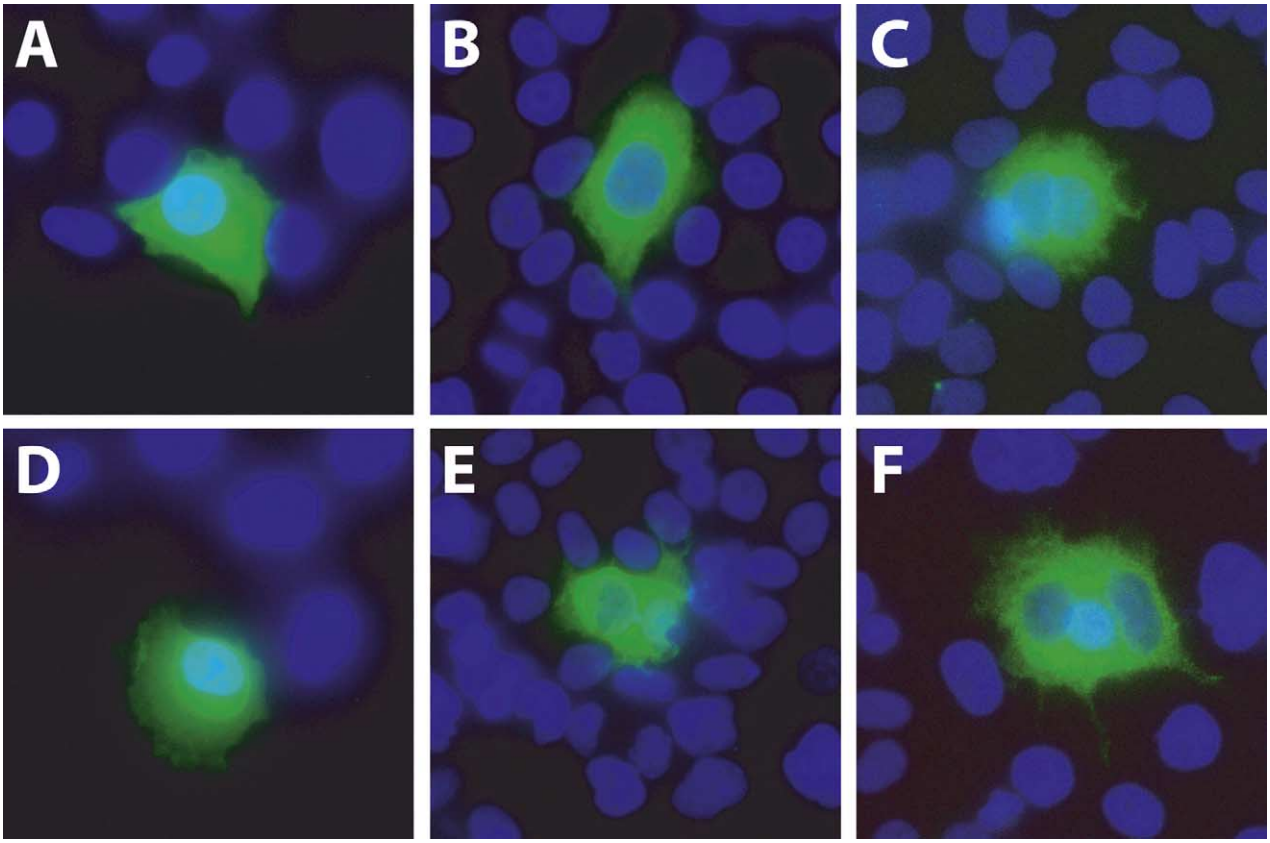


Fig. 4. Immunohistofluorescence analysis of the human and zebrafish PANE1-myc fusion proteins. The human (A–C) and zebrafish (D–F) PANE1-myc protein was located within the nucleus in non-confluent cultures (A,D), but it was excluded from the nucleus upon confluence (B,E). The protein was also excluded from the nucleus during cell division in both human (C) and zebrafish (F) PANE1-myc transfected cells. The myc tag is labeled in green and DAPI was used to stain the nuclei (blue).

produce stable cell lines we observed a silencing of the transgene and a selection of PANE1 non-expressing cells, suggesting that a high level of expression for a prolonged period is toxic.

This study revealed that PANE1 is preferentially expressed in a number of embryonic tissues as well as in activated B- and T-cells and lymphoid tumors. No expression was detected in cell lines of mammary origin. This suggests that the expression observed in mammary tissue transformed by a stabilized β-catenin (Renou et al., 2003) was the result of an immune response and invading lymphoid cells. The presence of a zebrafish ortholog, which has retained the characteristic sub-cellular distribution observed for mouse PANE1 suggests that this protein serves a function conserved in vertebrates. Although PANE1 lacks features found in transcription factors its nuclear localization suggests a role in transcription or maintenance of the genome.

3. Materials and methods

3.1. Multiple tissue expression array and Northern blot

A full-length human cDNA clone was obtained for the human PANE1 transcript (Invitrogen clone 3349507) and PANE1 was amplified using gene specific primers (F-5′ATGTCGGTGTTGAGGCCC3′ and R-5′CAGGTCC-TCCAGGGAGGG3′). The PCR product was purified (Qiagen) and then labeled with dCTP32 prior to hybridization. The human multiple tissue array 2 (Clontech 7776-1) and the human tumor multiple tissue Northern blot (Clontech 7757-1) were hybridized with the radiolabeled cDNA fragment for 2 h and then rinsed two times with 2 × SSC + 0.1%SDS. The blots were washed two times with 2 × SSC + 0.1%SDS for 15 min at 60 °C then washed two times with 1 × SSC + 0.1%SDS for 15 min at 60 °C. The blots were then placed at − 80 °C overnight with Kodak Biomax MR film to visualize the radioactive signal. Signal intensities were quantitated using the Alphaimage software. The tumor cell lines have been previously characterized (Collins et al., 1978; Chen, 1988; Lozzio and Lozzio, 1975; Minowada et al., 1972; Pulvertaft, 1964; Leibovitz et al., 1976; Trainer et al., 1988; Klein et al., 1968).

3.2. Cell cultures

Several cell lines were utilized for this study. Hela cells (Chen, 1988) were used for the transfections and they were raised in Dubelco's Modified Eagles Medium with 10% Fetal Calf Serum, penicillin, streptomycin and L-glutamine.

The B- and T-cells, MOLT-4 (Minowada et al., 1972) and Burkitt's lymphoma Raji cells (Pulvertaft, 1964) were cultured in RPMI-1640 with 10% Fetal Calf Serum, penicillin, streptomycin and L-glutamine. Mononuclear cells from peripheral blood (PBMC) or bone marrow (BMMC) of healthy donors were obtained by Ficoll Isopaque density centrifugation and cryopreserved in liquid nitrogen until further use. The PBMC were thawed and used directly for RNA isolation using Trizol (resting T-cells), or stimulated with phytohaemoglutanin and IL-2 to obtain activated T-cells, or with CD40 ligand-transfected L cells plus 4 ng/ml interleukin 4 to obtain activated B cells. Furthermore, immortalized B cells were obtained by Epstein Barr Virus (EBV) transformation using standard procedures. The SW480 cells were raised in Leibovitz's L-15 Medium with 10% Fetal Calf Serum, penicillin, streptomycin and L-glutamine. When the cells were frozen the normal medium was supplemented with 10% DMSO, frozen at −80 °C overnight and then placed in the liquid nitrogen cryosafe for preservation. Every procedure and aliquot used for analysis were performed or obtained in triplicate.

3.3. RNA preparation and first strand cDNA synthesis

RNA used from cell cultures was prepared using Trizol (Invitrogen) and further cleaned using RNeasy columns (Qiagen). Culture flasks (100 mm) were scraped and the cells were collected by centrifugation at 1000 rpm for 5 min at 4 °C. The medium was removed and the cells were placed on dry ice to facilitate lysis of the membranes. The cells were then thawed at room temperature and 2 ml of Trizol was added to each pellet. The cells were homogenized and the protocol from the manufacturer was followed precisely as directed for the rest of the procedure. The RNeasy clean-up after Trizol extraction was performed precisely as outlined in the manufacturer provided by the manufacturer. *D. rerio* RNA was also extracted using Trizol followed by the RNeasy clean-up. An entire adult zebrafish was homogenized in Trizol at a ratio of 0.1:1 (ml) of reagent. The manufacturers protocol was followed precisely as outlined. The RNeasy clean-up was performed precisely as outlined in the manufacturer's protocol. First strand cDNA synthesis was performed using the Superscript II cDNA synthesis kit as outlined by the manufacturer with the exception of multiplying the reaction volume to allow the use of 10 μg of RNA as a template.

3.4. RT-PCR

First strand cDNA was used from human cell culture and whole zebrafish samples. In addition to the cDNA samples transcribed in our laboratory we obtained a Human Tumor Multiple Tissue cDNA Panel composed of cDNA synthesized from various tumor models (Clontech K1422-1). The cell lines have been previously characterized (Hurst et al., 1993; Ahluwalia et al., 1986; Anderson, 1982; Biswas, 1984; Schmid et al., 1985; Chen, 1993; Kaighn et al., 1979). Primers were synthesized for Cyclophilin A (F-5′CTGCAGATGCTGTGTCTGGT3′ and R-5′CAGAAGGAATGATCTGGTGGT3′), and these primers were used to normalize all of the cDNA used for RT-PCR at 25 cycles and an annealing temperature of 58 °C. After all of the cDNA samples were normalized to 25 cycles they were then used to amplify PANE1 using the gene specific primers (F-5′ATGTCGTGTTTGAGGCCCC3′ and R-5′CAGGTCCTCCAGGGAGGG3′). The PANE1 RT-PCR was performed with an annealing temperature of 55 °C and 35 cycles.

3.5. Cloning and transfection

Whole adult zebrafish RNA was used as the template for synthesis of cDNA, and the PANE1 message was amplified using gene specific primers (F-5′CTCGAGTCGTGGTTGAAAGC-3′ and R-5′GGGAGATTATTTGGGCAACAC3′). The open reading frame (ORF) was amplified using primers that added Not1 and Xba1 restriction sites at the 5′ and 3′ ends respectively (F-5′ATAAGAATGCGGCCGCATGGCGCTCCTGTCGCCG3′ and R-5′CTAGTCTAGACTGCAGGTAGTCCTCCTGG3′; the R primer removes the endogenous stop codon). The human PANE1 cDNA was amplified from a full-length cDNA clone (Invitrogen clone 3349507) using primers that incorporated the Not1 and Xba1 restriction sites and removed the stop codon from the endogenous message (F-5′ATAAGAATGCGGCCGCATGTCGGTGTTGAGGCCCC and R-5′CTAGTCTAGACAGGTCCTCCAGGGAGGG3′). The resulting fragments were digested with Not1 and Xba1 (New England Bioloabs) and then ligated into the Not1 and Xba1 digested pcDNA4 version A expression vector in-frame with the myc/his tags. The *M. musculus* PANE1 expression vector has been previously described (Renou et al., 2003). The constructs were transfected using the Fugene reagent as instructed with a 3 μg of DNA to 9 μl of reagent in 60% confluent Hela cell cultures. The transfected expression vectors were allowed to incubate with the cells for 48 h before any further procedures were performed.

3.6. Immunofluorescence detection of the fusion proteins

Hela cells were transiently transfected with *M. musculus*, *H. sapiens* and *D. rerio* PANE1 vectors as stated above. After 48 h of incubation with the transfection reagent and respective vector the cultures were split and aliquots from the transfections (three replicates each) were incubated in serially diluted concentrations on chambered slides. The cells were allowed to grow overnight and then the medium was removed and the cells were fixed using 10% neutral buffered formalin for 30 min at room temperature. The cells were washed three times with 1 × PBS and then the membranes were permeablized with

0.1% Triton-x 100 diluted in 1 × PBS for 3 min at room temperature. The cells were then washed three times with 1 × PBS. The primary anti-myc antibody was diluted 1:1000 in 1 × PBS with 3% goat serum, and allowed to incubate at room temperature for 1 h. After incubation with the primary antibody the chambers were washed three times with 1 × PBS. The secondary goat anti-mouse alexafluor 488 conjugated secondary antibody was diluted in 1 × PBS with 3% goat serum added to each chamber and allowed to incubate for 45 min at room temperature while protected from exposure to light. After incubation with the secondary the chambers were removed from the slides and the slides were washed two times with 1 × PBS. The mounting medium containing DAPI was added to the slides, the cover slips were placed on the slides and then the fluorescence was visualized using an Olympus microscope with a mercury bulb for excitation.

Acknowledgements

The authors would like to thank Ling Zhao, Yongzhi Karen Cui and the members of LGP for technical support, critical review and necessary conversations relating to this study.

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